



Quantification of infectious HIV-1 plasma viral load using a boosted in vitro infection protocol

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Abstract

Methods currently used for HIV-1 viral load measurements are very sensitive, but cannot distinguish between infectious and noninfectious particles. Here we describe the development of a novel, sensitive, and highly reproducible method that allows rapid isolation and quantification of infectious particles from patient plasma.

By immobilizing HIV-1 particles in human plasma to platelets using polybrene, we observed a 10- to 1000-fold increase in infectivity over infection protocols using free virus particles. Using this method, we evaluated infectivity in plasma from 52 patients at various disease stages. At plasma viral loads of 1000–10 000 HIV-1 RNA copies/ml 18%, at 10 000–50 000 copies/ml 73%, at 50 000–100 000 copies/ml 90%, and above 100 000 copies 96% of cultures were positive. We found that infectious titers among patients vary distinctively but are characteristic for a patient over extended time periods. Furthermore, we demonstrate that by evaluating infectious titers in conjunction with total HIV RNA loads, subtle effects of treatment intervention on viremia levels can be detected. The immobilization procedure does not interfere with viral entry and does not restore the infectivity of neutralized virus. Therefore, this assay system can be utilized to investigate the influence of substances that specifically affect virion infectivity such as neutralizing antibodies, soluble CD4, or protease inhibitors. Measuring viral infectivity may thereby function as an additional, useful marker in monitoring disease progression and evaluating efficacy of antivirals in vivo.

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Introduction

HIV-1 particles in plasma represent the most recently produced quasispecies. Influences of the immune system or therapies on viremia can most rapidly be monitored by assessing this easily accessible compartment. Therefore, the quantification and characterization of the plasma virus pool is of particular interest for the diagnosis of HIV-1 infection and assessment of disease progression. Current methods routinely used for viral load measurements determine the amount of genomic HIV-1 RNA copy numbers but cannot distinguish between infectious and noninfectious or neutralized particles in plasma (Coombs et al., 1993; Mellors et al., 1997; Piatak et al., 1993b). Despite the high copy

numbers HIV-1 can reach in plasma, only a minor fraction of these particles has been described to be infectious (Andreoni et al., 1997; Coombs et al., 1989; Escaich et al., 1991; Ho et al., 1989; Lathey et al., 1994; Pan et al., 1993; Piatak et al., 1993a, 1993b; The Division of AIDS, National Institute of Allergy and Infectious Diseases and National Institute of Health, 1997; Vella et al., 2000). Direct co-culturing of patient plasma with donor cells is commonly used (Andreoni et al., 1997; Coombs et al., 1989; Escaich et al., 1991; Ho et al., 1989; Lathey et al., 1994; Pan et al., 1993; Piatak et al., 1993a, 1993b; The Division of AIDS, National Institute of Allergy and Infectious Diseases and National Institute of Health, 1997; Vella et al., 2000) but has proven to be rather insensitive (Table 1). Concentration of virus from patient plasma by centrifugation or precipitation before co-culturing the virus with target cells has been shown to increase the in vitro infectivity, but the necessary centrifugation and precipitation steps are time consuming, laborious, and prone to

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Table 1
Comparison of different virus isolation procedures

Reference	Assay short description	Number of successful virus isolations from plasma at different levels of HIV RNA copies/ml		
		< 10 000	10 000–100 000	>100 000
This work	virus cross-linking to platelets	1/12 (8.3%)	15/19 (78.9%)	20/21 (95.2%)
Piatak et al., 1993b	direct plasma PBMC co-culture	0/4 (0%)	2/21 (9.5%)	28/40 (70.0%)
Escaich et al., 1991	direct plasma PBMC co-culture	n.d.	n.d.	30/154 (19.5%)
Dictor et al., 2001	direct plasma PBMC co-culture	6/16 (37.5%)	7/14 (50%)	3/3 (100%)
Tedder et al., 1998	direct plasma PBMC co-culture	14/68 (20.6%)	2/6 (33.3%)	n.d.
Andreoni et al., 1992	direct plasma PBMC co-culture	n.d.	n.d.	15/22 (68.2%)
Vella et al., 2000	direct plasma PBMC co-culture	1/31 (3.2%)	1/21 (4.8%)	41/62 (66.1%)
Harrington et al., 2000	direct plasma PBMC co-culture	0/3 (0%)	2/6 (33.3%)	2/14 (14.3%)
Sarmati et al., 1994	polyethylene glycol treated plasma	n.d.	n.d.	30/42 (71.4%)
Ercoli et al., 1995	polyethylene glycol treated plasma	n.d.	n.d.	170/202 (84.2%)
Andreoni et al., 1997	polyethylene glycol treated plasma	n.d.	n.d.	62/86 (72.1%)

sample loss (Harrington et al., 2000; Sarmati et al., 1994; Sullivan et al., 1996). Nevertheless, virus isolations from plasma samples with a viral load under 100 000 HIV-1 RNA copies/ml have been only successful in a small fraction of analyzed samples irrespective of the isolation procedure used (Table 1).

To date, it remains unclear whether the bulk of plasma virions is indeed noninfectious (due to inactivation by immune defense or production of defective particles) (Dianzani et al., 2002; Piatak et al., 1993b; Sullivan et al., 1996) or if the inability to detect infectious particles in patient plasma is a consequence of the relative insensitivity of current methods used for virus isolation. The primary goal of the current study was to devise a sensitive assay system that allows quantification of infectious HIV-1 particles in plasma to define if the plasma virus pool carries a substantial infectious load and to which extent this pool of virions may participate in driving the infection in vivo.

Results

Polybrene induces effective capture of plasma-born HIV-1 to platelets

The primary intent of this study was to evaluate the infectious virus load in plasma of HIV-1-infected individuals. To this end, we sought to develop an improved assay strategy that allows reliable quantitation of infectious virus load in plasma.

It has long been realized that polycationic reagents such as polybrene can aid viral infection processes in vitro by increasing viral attachment (Le Doux et al., 2001; Manning et al., 1971; Pan et al., 1993; Toyoshima and Vogt, 1969). However, polybrene is highly toxic for cells and can be used only at concentrations below 20 µg/ml. Here we show that high concentrations of polybrene are substantially more effective in promoting virus capture to cells than conventionally used doses (Fig. 1). To be able to utilize the improved virion attachment at high, toxic polybrene concentrations, we adsorbed plasma virions in a first step to human platelets as “carrier” cells that are subsequently washed free of plasma and polybrene, and co-cultured with the actual target cells, activated primary CD4⁺ T cells. We chose human platelets as carrier cells for our assay development because these cells bind HIV-1 but are not known to support productive infection that should guarantee an optimal transmission to the desired target cells, primary CD4⁺ T cells (Lee et al., 1998, 1999; Youssefian et al., 2002). A further advantage of platelets is their small size and their higher surface to volume ratio compared to other cell types. As a consequence, high platelet numbers can be employed that should allow an optimal distribution of virions on individual cells, which is important for the quantification of infectious titers.

We found that after exposing human platelets for 4 h to seronegative human plasma spiked with either the R5 primary virus JR-CSF or the X4 primary virus 2044, only 16.8% of the R5 virus and 9.2% of the X4 virus had spontaneously bound to platelets as measured by deposition

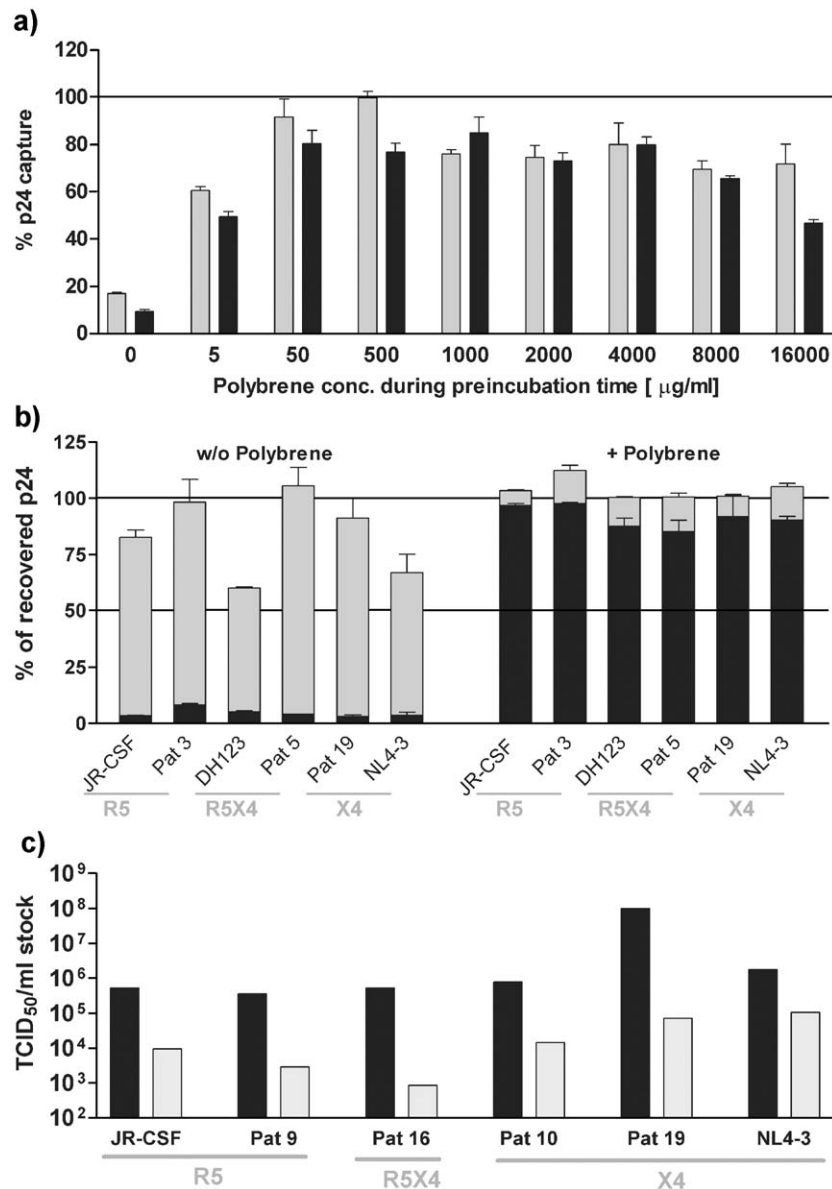


Fig. 1. Polybrene induces HIV capture to platelets and increases infectivity. (a) Seronegative plasma was spiked with 10 ng p24 of JR-CSF (grey bars) or 2044 (black bars) and reacted with platelets and increasing concentrations of polybrene for 4 h. Platelet-associated virus was then determined by measuring p24 antigen levels. Bars represent means of duplicate wells (\pm SD). Results from one of two experiments are shown. (b) Plasma was spiked with indicated virus isolates (10 ng p24) and incubated with platelets in the presence (right) or absence (left) of 2 mg/ml polybrene as described above. p24 antigen associated with platelets (black bar) or remaining unbound in the supernatant (grey bar) was determined. Bars represent means of duplicate samples. (c) Identical amounts of indicated virus stocks were titrated as free virus (grey bars) or after binding to platelets (black bars) on stimulated healthy donor CD8-depleted PBMC and TCID₅₀ determined.

of p24 antigen on the cells (Fig. 1a). However, by adding increasing concentrations of polybrene during the adsorption step, we were able to improve the capture efficiency to approximately 80% for both viruses. Maximum capture was reached at polybrene concentrations between 0.5 and 4 mg/ml.

Viral phenotype has no effect on capture efficiency

To rule out that our capture protocol selects for a specific viral phenotype, we evaluated the efficiency of

R5, R5X4, and X4 virus adsorption to platelets in the presence of polybrene (Fig. 1b). Seronegative plasma was spiked with the indicated HIV isolates and binding efficiency evaluated by measuring p24 antigen associated with platelets. No differences in the capture of R5, R5X4, and X4 viruses were observed. When polybrene (2 mg/ml) was added during the plasma incubation step, $91.4 \pm 5.0\%$ (SD) of the added p24 antigen was associated with platelets while $12.3 \pm 3.6\%$ (SD) of p24 antigen remained unbound in the supernatant. In contrast, when the adsorption was carried out in the absence of polybrene, only $4.6 \pm 1.9\%$

(SD) of the p24 antigen was associated with platelets and $79.4 \pm 17.6\%$ (SD) of p24 antigen remained unbound.

Platelet-captured virions are highly infectious

We next compared the in vitro infectivity of platelet bound to free virions using a panel of six virus isolates with different co-receptor usage (Fig. 1c). To define solely the impact of platelet-capture on infectivity, titrations were carried out in culture medium in the absence of human plasma. We found that platelet-immobilized virions were remarkably more infectious than free virus. The increase in infectivity upon immobilization to platelets ranged from 17- to 1413-fold (median = 90-fold; mean = 382-fold). It has been previously shown that positively charged compounds such as polybrene can potentially inhibit X4 virus infectivity (Haraguchi et al., 1997; Trkola et al., 1999). Considering the high concentrations of polybrene used for viral capture, it was therefore of importance to ensure that residual polybrene bound to platelets had no adverse effects on the infection process of X4 viruses. As depicted in Fig. 1c, we found no difference in the transmission efficiency between different viral phenotypes.

Thus, residual polybrene bound to platelets does not interfere with the infection process and virions are effectively captured and transmitted by polybrene-treated platelets irrespective of their co-receptor usage.

Timing and mode of virion capture to platelets

We next investigated the underlying mechanisms of the capture process induced by polybrene. We found that the polybrene-triggered binding of virions to the platelet membrane is a surprisingly rapid process (Fig. 2a). Within 15 min, already 65% of the R5-using virus Pat 5, and 66.5% of the X4-using virus Pat 19, had bound to the platelet membrane. Maximum capture was reached after 4 h. To probe if a specific interaction between viral envelope proteins and known receptors for HIV-1 occurs, we tested if a neutralizing antibody directed against the viral envelope proteins gp41 (2F5), the soluble CD4 molecule CD4-IgG2, which binds to gp120, or antibodies directed against CCR5 (PA14) and CXCR4 (12G5) inhibited virus binding to platelets. As shown in Fig. 2b, none of these reagents had an influence on virion capture induced by polybrene.

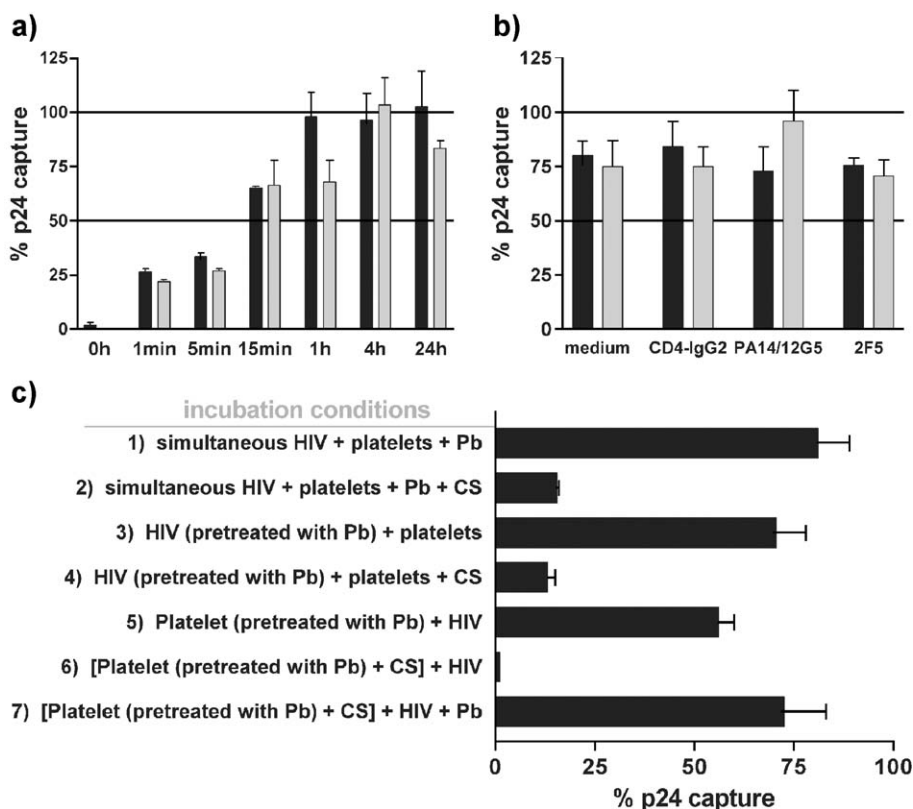


Fig. 2. Mechanism of polybrene-induced HIV binding to platelets. (a) The R5X4 isolate Pat 5 (black) and the X4 isolate Pat 19 (grey) were incubated with platelets in the presence of 2 mg/ml polybrene for the indicated time and p24 antigen associated with platelets determined. (b) Capture of JR-CSF (black) or 2044 (grey) to platelets was carried out either in medium alone or in the presence of 25 μ g/ml CD4-IgG2, 25 μ g/ml PA14 (for JR-CSF), or 50 μ g/ml 12G5 (for 2044), and 50 μ g/ml of Mab 2F5. Capture of virions to platelets was estimated by measuring p24 antigen. (c) Virus (SF162) was bound to platelets after addition of polybrene (2 mg/ml) and chondroitinsulfate (CS, 200 μ g/ml) at variable steps of the incubation procedure. Conditions 1–2: all reagents were added simultaneously. Conditions 3–4: virus was reacted with polybrene (2 mg/ml), after addition of platelets volume was increased to a final polybrene concentration of 10 μ g/ml. Conditions 5–7: platelets were first pretreated with polybrene, then washed, incubated with CS, and washed again before virus, and (in case of condition 7) polybrene was added.

Because polybrene is a highly positively charged compound, a postulated mechanism of its action in promoting virus adsorption is cross-linking of negative charges on virus and cell membranes (Donato et al., 1996; Le Doux et al., 2001). To probe if this is also the case for HIV-1 adsorption to platelets, we investigated if addition of negatively charged compounds can inhibit the effect of polybrene. To this end, we added the highly negatively charged glycosaminoglycan chondroitinsulfate (CS) at various steps of the binding process (Fig. 2c). When CS was added during the incubation of virus with platelets and polybrene, the capture of virions to platelets was reduced from $81 \pm 8\%$ to $15 \pm 0.5\%$ (SD). A similar reduction in capture efficiency was found when virus stocks, which were pretreated with polybrene, were allowed to bind to platelets in the presence or absence of CS. Equally, platelets pretreated with polybrene captured $56 \pm 4\%$ (SD) of virions in the absence of unbound polybrene in the medium. However, when these polybrene-pretreated platelets were exposed to CS, only $1 \pm 0\%$ (SD) of the input virus was captured. Of note, this low capture efficiency was reversed when polybrene was added back during the incubation with the virus. Taken together, these analyses strongly suggest that capture of virions to platelet membranes promoted by polybrene involves a cross-linking of negative charges on virion and cell membranes via the positively charged compound.

Assessment of virion capture from patient plasma by real-time PCR

Studying virion binding to cells by measuring p24 antigen deposition has limitations: not all p24 antigen in virus preparations is virion associated. Free p24 antigen is known to spontaneously bind to cell membranes (Marechal et al., 1998; Mondor et al., 1998) and might therefore influence the readout in the p24 antigen binding assay. Moreover, the sensitivity of the p24 antigen ELISA (1–10 pg/ml) is too low to perform direct binding studies with plasma from HIV-infected individuals. A viral load of 100 000 RNA copies/ml plasma corresponds to approximately 10 pg of p24 antigen (Chertova et al., 2002), and thus p24 antigen concentration in most patients samples would be under or close to the detection limit of the ELISA assay.

To verify our data obtained in the p24 antigen binding assay and to investigate if the virion capture procedure is also successful in retrieving virus from patient plasma, we measured virion adsorption to platelets by real-time PCR in plasma samples from 14 HIV-1 infected individuals (Fig. 3a and Table 2). The efficacy of virion attachment to platelets in the presence and absence of polybrene was evaluated and compared to the total number of RNA copies in the respective patient plasma. The latter was determined from viral pellets derived after high speed centrifugation of plasma. We found that polybrene induced a highly effective adsorption of HIV-1 to platelets. Quantities of virus measured after high speed centrifugation and after capture to polybrene-treated platelets

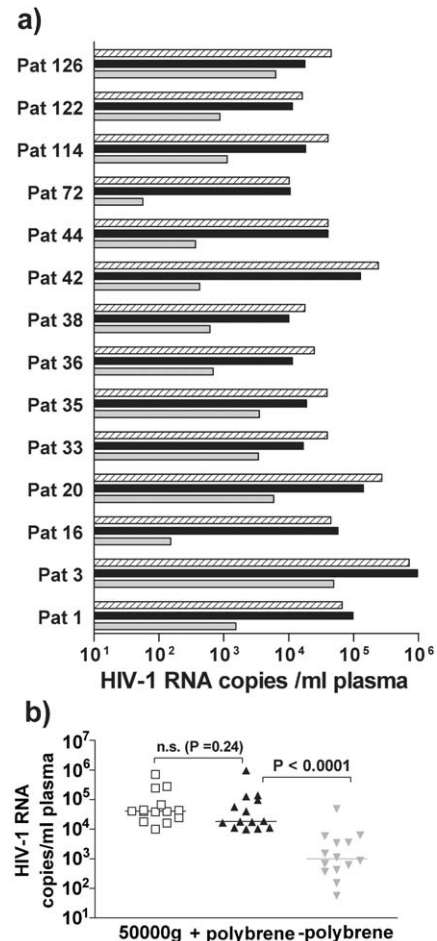


Fig. 3. Capture of HIV from patient plasma to platelets. (a) Virus from 14 plasma specimens was concentrated by high-speed centrifugation (hatched bars) or captured to platelets in the presence (black bars) or absence (grey bars) of polybrene and copies of viral RNA/ml plasma measured by real-time PCR. (b) Quantities of virus measured after high-speed centrifugation (open squares) and after capture to platelets with (full triangle) and without polybrene (open triangle) were compared using Wilcoxon signed rank test.

were not significantly different ($P = 0.2412$ Wilcoxon signed rank test; Fig. 3b). In agreement with our data obtained in the p24 antigen binding studies, we found that in the absence of polybrene, HIV-1 binding to platelets was significantly lower ($P < 0.0001$). Compared to RNA copy numbers after high-speed centrifugation, median recovery after capture to platelets in the presence or absence of polybrene were 95% and 5%, respectively.

Quantification of infectious HIV-1 plasma viral load using platelet-immobilized virions

We next compared the sensitivity of our new plasma virus titration assay with the commonly used protocol (The Division of AIDS, National Institute of Allergy and Infectious Diseases and National Institute of Health, 1997) that is based on direct co-culturing of plasma and target cells. Human plasma spiked with increasing concentrations of

Table 2
Quantification of infectious virus load in patient plasma

Patient	Age (years)	Gender	Clinical stage	Therapy	Isolation day	CD4 cells/mm ³	RNA (copies/ml)	Infectious titer (TCID ₅₀ /ml plasma)	Ratio infectious to total particles ^a	Co-receptor usage
1	32	m	A3	no	29.03.01	182	73,787	107.15	689	X4
2	49	m	C3	no	24.08.01	6	148,562	46.77	3176	R5X4
3	39	f	n.a.	no	05.04.01	298	116,844	107.15	1090	R5
5	37	m	C3	no	06.04.01	218	11,832	8.91	1328	R5X4
7	62	m	C3	no	09.04.01	16	232,421	398.11	584	R5
9	42	m	C3	no	17.04.01		146,220	26.92	5433	R5
10	52	m	n.a.	no	24.04.01	80	220,000	3.98	55262	X4
11	50	m	C3	no	12.06.01	63	75,840	61.66	1230	X4
13	40	m	C3	no	27.04.01	373	330,454	186.21	1775	R5
15	69	m	A3	no	07.05.01	210	62,926	3.98	15806	R5X4
16	36	f	C3	no	28.05.01	97	124,244	2.29	54234	R5X4
18	40	m	A2	no	28.06.01	488	131,431	275.42	477	R5
19	48	m	C3	no	02.07.01	188	73,889	20.42	3619	X4
20	40	m	A2	no	04.07.01	335	285,804	<2.29	n.a.	n.a.
29	39	m	A2	no	13.05.02	224	82,300	22.39	3676	R5
31	41	m	C3	no	14.05.02	431	86,900	5.25	16558	R5
33	40	m	A2	no	29.05.02	325	101,500	15.49	6553	R5
34	64	m	A3	no	15.07.02	64	144,000	186.21	773	n.i.
35	50	m	A3	no	15.08.02	312	105,000	5.25	20007	R5
36	36	m	A2	no	16.08.02	247	17,400	5.25	3316	R5
37	43	m	B1	no	19.08.02	417	30,700	8.91	3445	n.d.
38	31	m	n.a.	no	23.08.02	170	66,000	81.28	812	R5
39	36	m	A1	no	23.08.02	364	163,500	26.92	6075	R5X4
42	41	m	B3	no	12.09.02	197	178,500	35.48	5031	R5
43	41	m	A2	no	12.09.02	308	199,500	81.28	2454	R5
44	52	m	C2	no	19.09.02	196	115,000	20.42	5632	R5X4
47	46	m	A2	no	04.12.02	475	757	<2.29	n.a.	n.a.
48	35	m	C3	no	04.12.02	630	330	<2.29	n.a.	n.a.
51	36	m	B2	no	06.12.02	407	3130	2.29	1366	R5
53	42	m	A2	no	09.12.02	683	8170	<2.29	n.a.	n.a.
55	24	m	A1	no	13.12.02	464	2290	<2.29	n.a.	n.a.
60	36	m	A2	no	19.12.02	400	18,500	<2.29	n.a.	n.a.
61	27	m	A1	no	19.12.02	704	4470	<2.29	n.a.	n.a.
63	26	m	A3	no	06.01.03	76	12,800	7.94	1611	R5X4
64	40	m	A2	no	13.01.03	338	13,900	7.94	1750	R5
65	40	m	A1	no	16.01.03	410	40,900	<2.29	n.a.	n.a.
66	35	f	A3	no	31.01.03	197	2930	<2.29	n.a.	n.a.
67	23	m	B3	no	19.02.03	9	389,000	416.87	933	n.i.
68	40	m	A2	no	19.02.03	328	64,650	<2.29	n.a.	n.a.
69	31	m	B3	no	20.02.03	50	1,310,000	416.87	3142	R5
70	29	m	C3	no	24.02.03	32	815,000	6.92	117803	X4
71	50	m	B3	no	27.02.03	113	144,000	6.92	20814	R5
72	41	m	B3	no	28.02.03	104	155,000	6.92	22404	R5
107	48	f	A2	no	18.12.02	382	2250	<2.29	n.a.	n.a.
113	62	m	B2	no	07.11.02	629	74,900	26.92	2783	R5
114	33	m	A1	no	17.06.03	811	17,603	3.38	5208	R5
115	36	m	A1	no	10.12.02	625	5490	<2.29	n.a.	n.a.
117	37	f	A2	no	19.12.02	336	4560	<2.29	n.a.	n.a.
121	40	f	A2	no	18.12.02	242	11,200	<2.29	n.a.	n.a.
122	44	m	A2	no	04.12.02	511	43,400	2.29	18945	R5
125	37	f	A2	no	16.12.02	750	2350	<2.29	n.a.	n.a.
130	70	m	A1	no	06.12.02	768	<50	<2.29	n.a.	n.a.

^a Corresponds to TCID₅₀ per two RNA copies.

n.d., not done; n.a., not applicable.

the R5 isolate JR-CSF was titrated using both protocols. As depicted in Fig. 4a, the new assay yielded a 1–3 log higher infectivity at all input concentrations tested. While cultures in the direct plasma co-cultures remained negative up to an input of 30 pg of virus (measured as p24 antigen), the

platelet-cross-linked samples were positive even at the lowest concentration tested (0.3 pg). We found a mean 333-fold increase in sensitivity over the traditional procedure when virion capture to platelets was performed with isolates JR-CSF, DH123, and 2044 as representatives of R5,

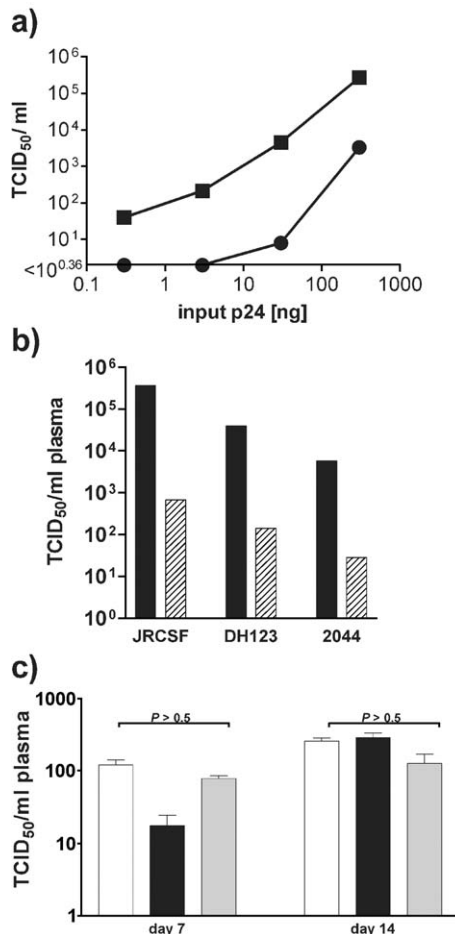


Fig. 4. Platelet cross-linking increases sensitivity of plasma titration. (a) Plasma spiked with the indicated concentration of the R5 virus JR-CSF was titrated using the standard plasma-co-culture protocol (circles) and compared to spiked plasma titrated by the platelet cross-linking protocol (squares). One of three independent experiments is shown. (b) Comparison of TCID₅₀ of plasma spiked with isolates JR-CSF (121.1 pg), DH123 (2.7 pg), and 2044 (1.8 pg) after using the standard plasma-co-culture protocol (hatched bars) and the platelet cross-linking protocol (black bars). (c) Plasma spiked with virus JR-CSF (1.8 pg p24/ml) was titrated using the platelet cross-linking protocol on three different target cell preparations (donors A, B, and C). Bars depict means of three titration assays per donor cells. TCID₅₀ on days 7 and 14 was compared using ANOVA (Kruskal–Wallis test with Dunn’s multiple comparison test).

R5X4, and X4 using virus isolates, respectively (Fig. 4b). To evaluate the reproducibility of the new plasma titration protocol, seronegative human plasma was spiked with the R5 isolate JR-CSF and stored in aliquots at -80°C . The spiked plasma was then titrated on three different donor cell preparations. As shown in Fig. 4c, on day 7 postinfection, the measured TCID₅₀ in three independent assays showed some variation that is not unexpected due to the known variability of primary donor cells in supporting HIV infection. However, these differences were not statistically significant and influences of the donor cells on the determination of infectious titers were negligible after 14 days of culturing. Taken together, our novel assay provides

a 1–3 log increase in sensitivity over traditional protocols by boosting the viral attachment process and diminishing cytostatic effects of plasma on target cells. Equally important, the assay is highly reproducible and due to its sensitivity allows a reliable quantification of infectious units in frozen plasma samples.

Virus isolated from plasma represents current quasiespecies

Autologous patient virus is commonly isolated from PBMC because isolations from plasma have in general been less successful (The Division of AIDS, National Institute of Allergy and Infectious Diseases and National Institute of Health, 1997). However, sequence analysis of virus quasiespecies present in plasma and of virus isolates derived from PBMC has shown that viral isolates derived upon activation of cells do not always reflect the most recently produced virus strains (Gunthard et al., 1998; Havlir et al., 1996; Wei et al., 1995). To validate our virus isolation protocol, we isolated virus from three patients at several time points from PBMC and from plasma using our new protocol. We then compared viral envelope sequences of these viral isolates with envelope sequences derived directly from plasma and PBMC to probe which isolation procedure represents best the actively replicating virus population (Fig. 5). Fifteen to 16 individual clones derived after RT-PCR of viral RNA from each specimen were available for phylogenetic analysis. Sequences that represented clusters within one patient were grouped and distribution of these clusters in plasma and PBMC specimen analyzed. Patient 113 was infected with a rather heterogeneous virus population. Nonetheless, clustering did not reach significance level. Sequences spanning the C2-V3 region belonged to the same quasiespecies in all specimen analyzed at the first time point and the same virus population was also dominant at the second time point analyzed. We also found a dominant viral quasiespecies in the plasma of patient 120 (13 out of 16 clones), which was the sole viral population isolated from both plasma and PBMC cultures. Net divergences between plasma and plasma culture as well as between plasma and PBMC culture were very low in these two patients (range 0.0–0.4%). The most variable virus population was found in patient 109. Several virus quasiespecies were present in plasma and plasma culture at the earliest time point analyzed. However, net divergence was only 0.7%. Noteworthy, between the first and second time point analyzed, this patient had been on antiretroviral treatment, which was interrupted 2 weeks before the second time point (Oxenius et al., 2002). At that time, the population emerging in the plasma was also isolated from plasma culture but not from PBMC culture. Net divergence from plasma was 1.3% and 4.7% for plasma culture and PBMC culture, respectively. Five months later, a different quasiespecies was dominant in plasma that had already been present in the earliest plasma sample analyzed. Again, a matching population was well represented in the plasma culture isolate (divergence 0.01%). At the last time point analyzed for patient 109, identical virus

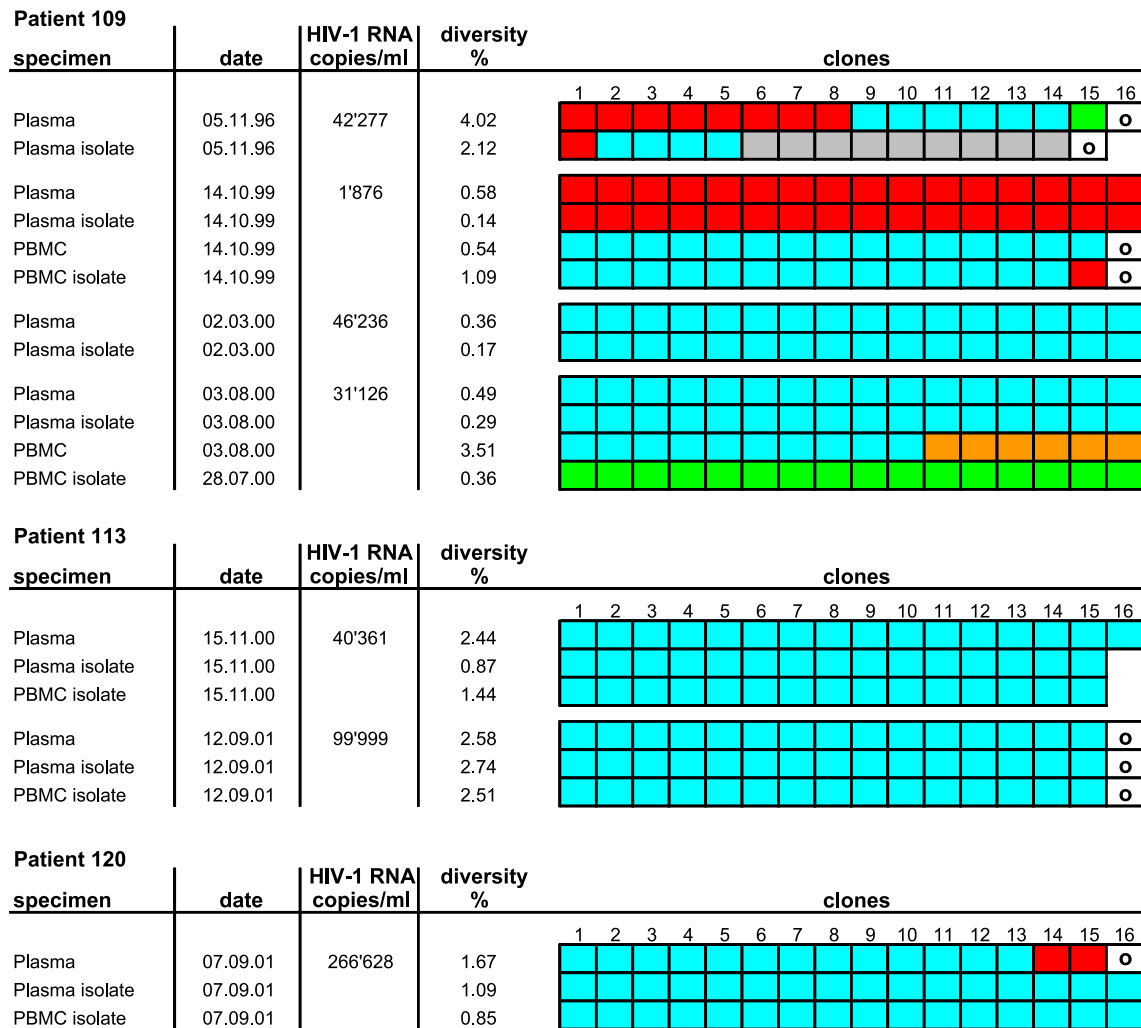


Fig. 5. Phylogenetic analysis. Phylogenetic analyses of HIV-1 envelope sequences encompassing the C2 and V3 domains from 15 to 16 individual clones derived after RT-PCR of viral RNA that was obtained directly from plasma and patient PBMC or from viral isolates derived from plasma and PBMC cultures were performed using the software package PHYLIP version 3.6. Samples from three patients were analyzed separately (Table 2). Boxes indicate the individual clones. Sequences that clustered with quasiespecies present in one patient are shown in the same color code. Blank boxes with a circle indicate single clones that differed significantly from other quasiespecies found within this patient.

species were found after direct sequencing of plasma and in the virus population isolated from plasma. However, the virus isolate derived from PBMC after stimulation of cells did not represent clones that were actively replicating at this time point but likely reflected a reactivated ancestral strain. The corresponding net divergences were 0.06% for the plasma culture and 3.9% for the PBMC culture, respectively.

Taken together, our isolation procedure yields virus isolates that on large represent the current replicating virus pool in plasma whereas, as described previously, virus isolation from PBMC can lead to the reactivation of latent, ancestral strains.

Infectious virus load at different disease stages

We next determined the efficiency of our procedure in isolating infectious virus from plasma of 52 patients at

various disease stages (Table 2). Plasma virus loads of analyzed samples ranged from <50 to 1 310 000 RNA copies per ml (median = 73 838; mean = 124 465). We successfully isolated plasma virus in 36 out of the 52 samples analyzed. The overall success rate in isolating virus from plasma was 69%. No virus could be isolated at viral loads below 1000 copies/ml (zero out of three samples). Eleven percent (1/9) of plasma samples with 1000–10 000 RNA copies/ml, 70% (7/10) of samples with 10 000–50 000 copies/ml, 89% (8/9) at loads between 50 000–100 000 copies/ml and above 100 000 copies/ml 95% (20/21) of samples yielded positive cultures. None of the previously published plasma isolation assays had a comparable sensitivity in isolating infectious virus from high and low viral load plasma (Table 1).

In a next step, we determined the infectivity (TCID₅₀) of these plasma samples (Table 2) and investigated the

relationship between infectious virus load and total virus load as measured by RNA copies in plasma. Infectious doses ranged from <2.29 to 417 TCID₅₀/ml plasma and correlated strongly with the total viral load (Fig. 6). Correlations remained significant when only samples that gave a positive result in the infectivity assay were used. Both total and infectious viral load correlated inversely with the CD4 count ($r^2 = 0.4041$, $P < 0.0001$ and $r^2 = 0.2694$, $P < 0.0001$, respectively). No disparity in infectious titers obtained from patients infected with R5 or X4 was observed. Viral RNA copies per TCID₅₀ ranged from 477.2 to 117 803 (mean 11 550 copies/TCID₅₀). Up to now it has been unclear whether the low infectivity measured in patient plasma reflects accurately the in vivo situation or is a result of inadequate assay systems. Using this boosted in vitro culturing system, we found a higher degree of infectivity in patient plasma than previously reported. Nevertheless, infectious titers remained low compared to the total amount of viral RNA copies detected. Thus, even with our boosted infection procedure, HIV particles in plasma are predominantly noninfectious.

Virus infectivity and antivirals

Low infectious to noninfectious particle ratios may reflect the production of genetically defective particles (Piatak et al., 1993b) or be a result of active immune defense. So it has been shown that the majority of HIV-1 particles found in patient plasma is immune complexed by antibodies and complement (Dianzani et al., 2002; Stoiber et al., 2001). To what extent these immune complexed viruses remain infectious is currently not known, but it is feasible that differences in infectious titers between patients could in part be due to differences in the activity

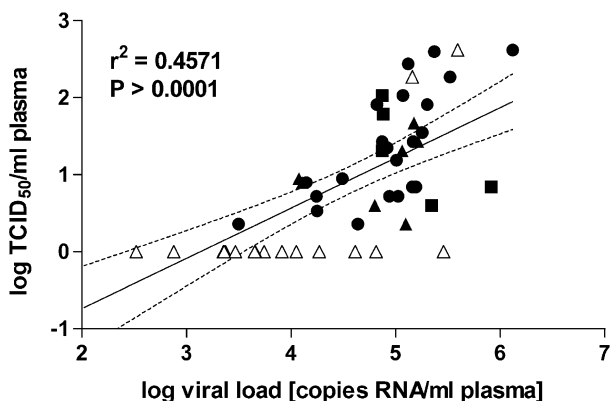


Fig. 6. Characterization of infectious virus pool in patient plasma. The infectious titer (TCID₅₀) of plasma samples from 51 chronically infected patients (see Table 2) was determined using the platelet-cross-linking protocol. Correlation analysis of log TCID₅₀ in plasma and log virus load (total number RNA copies per ml) is shown. Co-receptor usage of isolates is indicated R5 user (circle), X4 user (square), R5X4 user (closed triangle), isolates with undetermined co-receptor usage (open triangle).

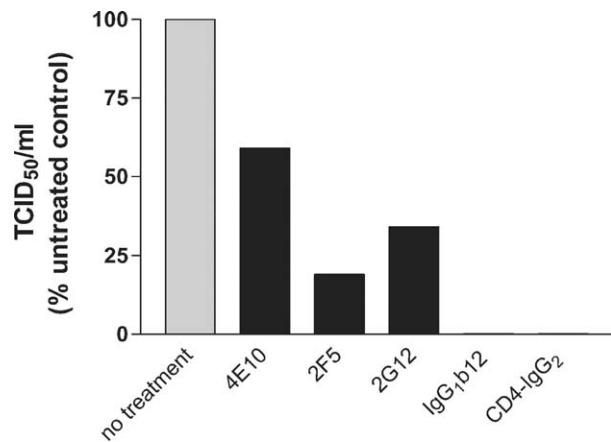


Fig. 7. Immune complexes stay intact during plasma titration. Virus (SF162, 150 TCID₅₀) was incubated with the neutralizing anti-gp41 Mabs 2F5 and 4E10 (50 µg/ml each), the neutralizing anti-gp120 Mabs 2G12 (50 µg/ml) and IgG1b12 (10 µg/ml), and the tetrameric CD4 molecule CD4-IgG2 (50 µg/ml) for 1 h at 37 °C. Immunocomplexed viruses were then titrated using the platelet-cross-linking protocol. One of two independent assays is shown. The percentages of infectious titer (TCID₅₀) obtained in the untreated control are depicted.

of autologous neutralization antibodies. Of note, we found that virus treated with neutralizing antibodies or tetrameric CD4-IgG2 before cross-linking to platelets remained non-infectious (Fig. 7), indicating that immune complexes stay intact during the cross-linking process. Therefore, it is possible to use our method to evaluate the in vivo impact of neutralizing antibodies or other antiretrovirals that affect virion infectivity directly.

To explore to what extent viral infectivity fluctuates over extended time periods, we analyzed infectious titers and total plasma viral load in three chronically HIV-1-infected patients over a time period of 3–4 months. We found that ratios between total and infectious viral load varied considerably between patients (Table 2), but were characteristic for a patient over extended time periods (Fig. 8). We further determined the infectious titer of four patients harboring partially ART-resistant HIV who received in addition to a combination of reverse transcriptase and protease inhibitors the fusion inhibitor T-20 (Table 2 and Fig. 9). Upon receiving T-20, patients 9 and 11 decreased viral loads by several logs. Concurrently, infectious titers dropped to undetectable levels in both patients. Only a moderate reduction in viral load (0.95 logs) was observed in patient 19 that was reflected by a 2.95-fold decrease in infectious titer. Interestingly, patient 7 failed to reduce viral loads in response to T-20 treatment. However, while total viral loads were maintained, viral infectivity showed a decline from 398 to 27 TCID₅₀/ml and CD4 cell levels increased from 16 to 34, indicating that this patient's virus was not completely resistant to T-20. These findings underline the potential of monitoring plasma infectivity in parallel to total viral

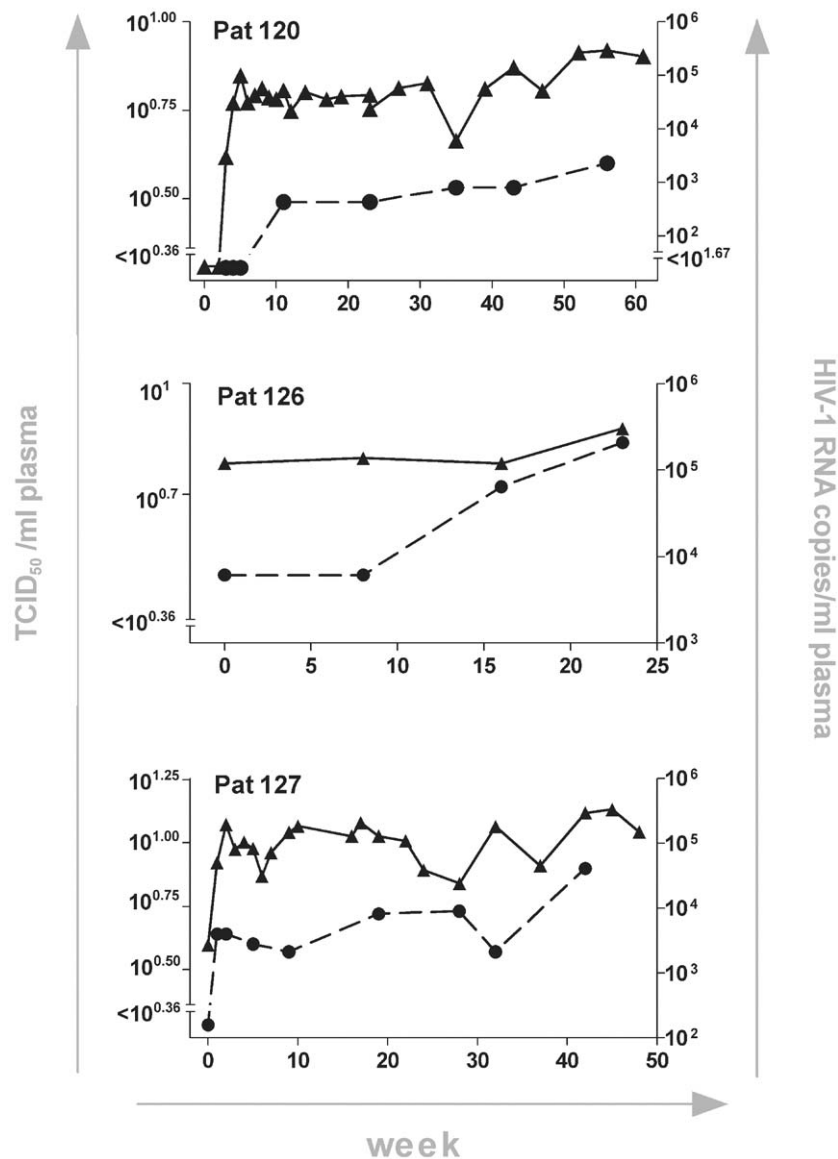


Fig. 8. Longitudinal evaluation of infectious virus load in patient plasma. The infectious titer (TCID₅₀) of plasma samples from three chronically infected patients (see Table 2) was determined using the platelet-cross-linking protocol. TCID₅₀ per ml plasma (circles) and virus load (total number RNA copies per ml; triangles) are shown.

load, particularly when evaluating the efficacy of anti-virals that target virions directly and modulate their infectivity.

Discussion

Quantitation of virus particles in patient plasma is due to the easy accessibility of this compartment and the sensitivity of the assays, the commonly evaluated virologic marker for the assessment of disease progression and effects of therapeutic intervention. However, current methods routinely used for viral load measurements determine the amount of genomic HIV-1 RNA copy numbers but cannot distinguish between infectious and noninfectious or neutralized par-

ticles in plasma (Coombs et al., 1993; Mellors et al., 1997; Piatak et al., 1993b).

The infectious virus load in plasma of HIV-1-infected individuals is traditionally measured by co-culturing serial dilutions of patient plasma with stimulated healthy donor PBMC (Andreoni et al., 1997; Coombs et al., 1989; Escaich et al., 1991; Ho et al., 1989; Lathey et al., 1994; Pan et al., 1993; Piatak et al., 1993a, 1993b; The Division of AIDS, National Institute of Allergy and Infectious Diseases and National Institute of Health, 1997; Vella et al., 2000). However, virus isolations from patient plasma have been on large less successful than virus isolations from cellular material. Despite high viral copy numbers in plasma, only a minor fraction of these HIV-1 particles were described to be infectious (Dianzani et al., 2002). Whether

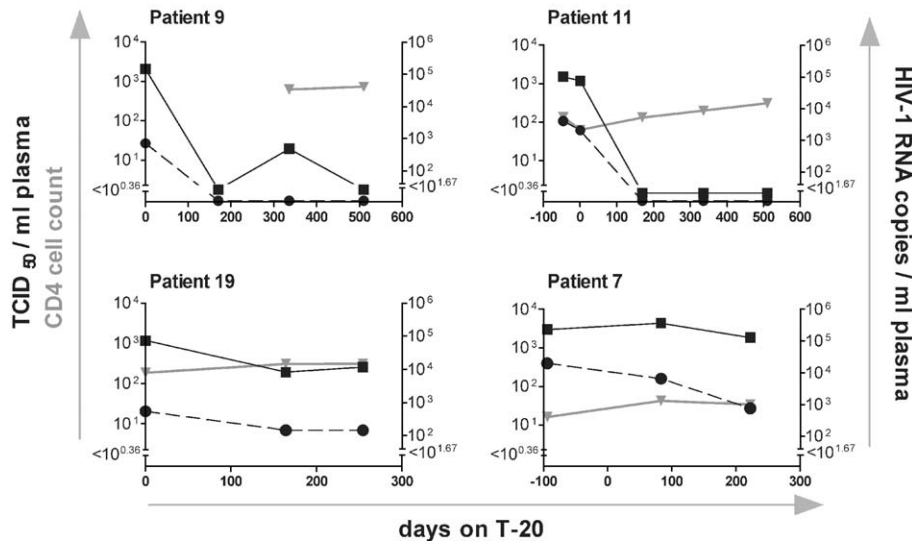


Fig. 9. Infectious virus load during antiretroviral treatment. The TCID₅₀ of plasma from four chronically infected patients (see Table 2) receiving antiretroviral treatment in combination with the fusion inhibitor T-20 was determined. TCID₅₀ per ml plasma (circles), virus load (total number RNA copies per ml; squares), and absolute CD4 T cell levels per mm³ blood (triangles) are shown.

this is due to insensitive assay systems or if indeed the majority of the virions found in plasma are noninfectious particles remains uncertain. The objective of the present study was to develop a reliable and sensitive protocol for the quantification of HIV-1 virus particles from patient plasma that allows to investigate the relative contribution of the plasma virus pool in maintaining and spreading the infection.

There are numerous technical factors that limit the success of virus isolation from plasma. Foremost, plasma at high concentration can clot upon co-cultivation with target cells, and this gel formation can substantially diminish infectability by reducing cell viability and motility in the culture system (Lathey et al., 1994; Pan et al., 1993; The Division of AIDS, National Institute of Allergy and Infectious Diseases and National Institute of Health, 1997) and data not shown). Secondly, residual antiviral factors in patients plasma such as neutralizing antibodies, complement, chemokines, cytokines, or drugs can affect the success of virus isolation (Lathey et al., 1994; Pan et al., 1993). Lastly, as with all assays that utilize human PBMC as target cells, a considerable variability due to differences in infectability among donor cells can occur.

Entry of HIV-1 into host cells requires binding of the viral envelope protein to the primary receptor CD4 and a fusion co-receptor, which belongs to the chemokine receptor family, most commonly the chemokine receptor CCR5 and CXCR4. Besides these entry receptors, several other cell surface molecules such as adhesion molecules (Fortin et al., 1997), glycosaminoglycan side chains of proteoglycans (Mondor et al., 1998; Ohshiro et al., 1996; Patel et al., 1993; Saphire et al., 2001; Ugolini et al., 1999), and lectins (Geijtenbeek and van Kooyk, 2003) have been identified that function as auxiliary receptors aiding viral attachment

to target cells by interacting with either viral- or host-derived proteins on the virion surface.

The rate-limiting step in the penetration of target cells by HIV-1 is considered to be the attachment of the virion to the cell surface, not the subsequent binding to the receptors or the fusion reaction (Orloff et al., 1991). Accordingly, it has been shown that by increasing viral attachment by employing centrifugal forces, in vitro infection rates can be substantially boosted (Ho et al., 1993; O'Doherty et al., 2000; Pietroboni et al., 1989). However, the necessary centrifugation steps are time consuming and do not allow for processing of larger sample numbers.

The essence of the method introduced here is that virus particles are bound to carrier cell membranes that allow a quick and reliable separation of virus particles from plasma. Thereby we achieve the following: (i) the infection process occurs in the absence of patient plasma. Thus, factors present in plasma that inhibit infection are removed. (ii) Virus particles are cross-linked to cell membranes which increase sedimentation rates compared to cell-free virus preparations and thereby aid the attachment and subsequent infection process.

The assay utilizes the polycationic sugar polybrene to cross-link virions to platelets as carrier cells. Subsequent culturing of immobilized virus with stimulated healthy donor PBMC yields 1–3 log higher infection rates than direct co-culturing of infectious plasma with target cells. Binding of virions to platelets induced by polybrene does not involve interaction with CD4 and co-receptors nor did we observe a direct involvement of the viral envelope proteins in this process. We provide evidence that the positively charged compound polybrene cross-links negative charges on virion and cell surface and thereby promotes viral attachment. This cross-linking process is highly

efficient, and 80–100% of virus particles can be captured by this method. Of note, we have also found that the same method can be used to increase infectivity of other retroviral vectors, irrespective of which cellular receptors are utilized for entry (data not shown). Most importantly, the cross-linked virions are highly infectious and are efficiently transferred to susceptible target cells. We found that platelet immobilized virions are 1–3 logs more infectious than free virus particles. The degree of infectivity enhancement seen was fairly consistent when samples from a specific individual were measured but varied among patient isolates. Therefore, the assay will be most useful for longitudinal monitoring of intra-patient changes in viral infectivity.

A specific advantage is that using our new method, infectious virus can be isolated from patient plasma with a higher success rate than described with previously reported assay systems (Table 1). As noted above, a general problem in all assay types that employ primary human PBMC as target cells is the vast variability in susceptibility to HIV infection among donor cells. Here we show that using cell pools from three donors in combination with our enhanced infection protocol allows a reliable and highly reproducible quantification of virus titers in patient plasma. Due to its increased sensitivity, the assay also allows a reliable quantification of the infectious virus load in frozen plasma samples. We have also used the same virus capture method to increase infectivity of virus on various cell lines with a similar success in boosting infectivity (data not shown).

Up to now, replication-competent, infectious virus is more frequently isolated and quantified from patient PBMC because virus isolations from plasma have been on large less successful (Pan et al., 1993; The Division of AIDS, National Institute of Allergy and Infectious Diseases and National Institute of Health, 1997). Although the number of infected cells in the periphery reflects cell-free viral loads under ongoing viral replication, isolating virus from patient cells bears the risk of reactivating latent, ancestral viral strains. So it has been shown that viral isolates derived upon activation of cells do not always yield the most recently produced virus isolates and appearance of mutant viruses in plasma may precede the manifestation of these quasispecies in cells of the periphery by several weeks (Gunthard et al., 1998; Havlir et al., 1996; Wei et al., 1995). Particularly when studying responses to novel therapies, a rapid and exact determination of escape mutants is critical and methods that allow a reliable and sensitive isolation of viruses from plasma are therefore sought for. Here we show that our novel isolation procedure successfully derives replication-competent virions from plasma. The isolated quasispecies reflected the dominant viral variants present in the patient's plasma at the given time point whereas virus isolated from PBMC can include reactivated ancestral variants. Given the increased sensitivity in both virus isolation and quantification, our assay may become a useful tool in monitoring effects of novel drug regimens and vaccination strategies on virion infectivity. Measuring effects

on virion infectivity might be particularly informative when effects of substances that target virions directly, such as neutralizing antibodies, are tested.

Most importantly, our newly developed assay allowed us to get insights into the infectivity of virions in human plasma *in vivo*. Little is known about the ratio of infectious to noninfectious virus in plasma of HIV-1-infected patients and how this ratio evolves throughout pathogenesis. Here we show that infectious to noninfectious ratios vary substantially between patients but are characteristic for an individual for extended time periods. It has been suggested that the majority of virions present in plasma have been actively combated by the immune system and are complexed by antibodies and complement (Dianzani et al., 2002; Stoiber et al., 2001). To what extent these immune complexed viruses maintain their infectivity is not known, and whether a high disparity between infectious titers and total viral loads reflects higher degrees of immune defense or production of defective particles bears further investigation. Our data indicate that the immobilization procedure used in our assay does not interfere with viral entry and does not restore the infectivity of neutralized virus. Thus, this assay allows an accurate quantitation of infectious particles. Due to the rapid replication and the high mutation rate of HIV-1, a substantial proportion of the virus particles in plasma will also comprise defective virions (Perelson, 2002). It has been a longstanding debate whether HIV-1 spread *in vivo* occurs mainly through cell–cell or free virus–cell transmission. Recent findings have shown that viral transmission *in vivo* occurs between cells near the so-called viral synapses (Jolly et al., 2004; McDonald et al., 2003; Zhang et al., 2004). To what extent replication-competent, infectious particles are present in plasma and contribute to the maintenance of the infection *in vivo* is not known. Virus production rates of infected cells in the periphery have been estimated to be in the range of 100–10 000 RNA copies per infected cell per day (Funk et al., 2001; Hockett et al., 1999; Stafford et al., 2000). Here we show using our boosted infection assay that viral infectivity in plasma is indeed exorbitantly low and that on average only one infectious dose per 5775 viral particles (11 550 HIV-1 RNA copies) is found. It is possible that our assay may still underestimate the *in vitro* infectivity of patient plasma and that more sensitive assays may be devised. However, when taking our data on viral infectivity as measure, this would mean that on average less than one infectious unit is produced by an infected cell. Because it cannot be expected that each infectious particle will succeed in infecting a new target cell, it is unclear how the infection *in vivo* would be maintained on such low infectivities given the infection process *in vivo* indeed depends on viral transmission in plasma. There are several possible explanations to this finding: firstly, should free virus transmission in plasma play a substantial role *in vivo*, then this can only be the case if either viral infectivity is higher than measured in our assay or alternatively, viral productions rates are

substantially higher than previously suggested. Secondly, it can be speculated that the viral compartment in plasma may only play a subordinate role in vivo and that as previously suggested cell–cell transmission in infected tissue is indeed more efficient in driving viral spread in vivo (Jolly et al., 2004; McDonald et al., 2003; Zhang et al., 2004).

Materials and methods

Reagents

MAbs 2F5 (Muster et al., 1993) and 2G12 (Trkola et al., 1996) were gifts from H. Katinger. Mab IgG₁b12 (Burton et al., 1994) was provided from D. Burton. Mab 12G5 was from Pharmingen and the CD4-IgG₂ molecule and Mab PA14 were from P. Maddon (Allaway et al., 1995). The following reagents were purchased from Sigma: Polybrene (Hexadimethrine bromide), HEPES buffer, chondroitin sulfate (CS), phytohemagglutinin (PHA).

Stimulated CD8 depleted PBMC

Buffy coats obtained from three healthy blood donors were depleted of CD8⁺ T cells using Rosette Sep cocktail (StemCell Technologies Inc.) and PBMC isolated by Ficoll-Hypaque centrifugation. Cells were adjusted to 4×10^6 per ml in culture medium (RPMI 1640, 10% FCS, 100 U/ml IL-2 (NIAID repository), glutamine, and antibiotics), divided into three parts, and stimulated with either 5 µg/ml PHA, 0.5 µg/ml PHA, or anti-CD3 Mab OKT3. After 72 h, equal numbers of CD8-depleted PBMC stimulated by one of these three methods were combined and are called stimulated CD8-depleted PBMC.

Platelet preparation from healthy donors

Platelets were obtained from HIV-negative, healthy blood donors. Blood was collected into EDTA vacutainer vials (Becton Dickinson) and centrifuged at $550 \times g$ for 15 min. The top layer containing platelet-rich plasma was collected and platelet counts determined using an AcT diff analyzer (Beckman Coulter). Platelet-rich plasma was then aliquoted in 1.5 ml Sarstedt tubes, stored at -70°C , and subsequently used as source of platelets.

Plasma collection from patient blood

Plasma sampled from patients at various disease stages was included in the current study (Table 2). Unless otherwise stated, these patients had not received antiviral therapy for extended time periods before blood sampling due to various clinical reasons. Informed consent was obtained from all patients according to the guidelines of the University Hospital Zurich. Patient blood was sampled in EDTA vacutainer vials. Within 6 h after blood draw, platelet-rich plasma was

collected and frozen in 1-ml aliquots at -70°C . We preferred using frozen plasma because fresh plasma causes the human platelets added to cross-link virions to form aggregates that are difficult to resuspend and might therefore not allow accurate titration of the infectious particles. Four patients received a triple combination of antiviral drugs and T-20 (Fig. 9). These individuals and patients displayed in Fig. 8, where plasma sampling was not performed as described above, were not included in the analysis depicted in Fig. 6 and Table 1.

p24 antigen ELISA

HIV-1 p24 antigen concentrations were measured by an in-house p24 antigen enzyme-linked immunosorbent assay as described (Moore et al., 1994; Trkola et al., 1995).

Virus stocks

The origin of virus isolates JR-CSF (R5), DH123 (R5X4), 2044 (X4), and NL4-3 (X4) has been described (Adachi et al., 1986; Koyanagi et al., 1987; Shibata et al., 1996; Simmons et al., 1996). Virus isolates Pat 3 (R5), Pat 19 (X4), Pat 5 (R5X4) Pat 109 (R5), Pat 113 (R5), and Pat 120 were obtained from patient CD4⁺ T cells as described (Trkola et al., 2003; Wong et al., 1997). Stocks from these viruses and viruses isolated from plasma were grown and titrated on CD8-depleted PBMC. The 50% tissue culture infectious dose (TCID₅₀) of virus stocks was determined by performing serial 5-fold dilutions of 100 µl viral inoculum in eight replicates in a 96-well tissue culture plate and calculated using the method of Reed and Muench (1938). In Fig. 1c, the same method was used to determine the impact of platelet immobilization on viral infectivity. In this case, equal amounts of virus inoculum either in solution or immobilized to platelets were used. Co-receptor usage of all virus isolates (see Table 2) was determined as described (Trkola et al., 1998).

Isolation and titration of HIV-1 from plasma samples

Thawed patient plasma was depleted from autologous platelets and a constant number of donor platelets were added to provide similar binding surfaces in all samples. Both too low and too high carrier platelet numbers had adverse effects on the isolation efficiency: low carrier platelet numbers resulted in reduced virus capture whereas too high numbers of platelets in the co-cultures were toxic for the target cells (data not shown). HIV-1 in vivo can bind spontaneously to platelets although to a low extent (data not shown). Thus, removing autologous platelets before virus isolation is of little consequence on the total virus load in the plasma sample. Platelet-rich patient plasma (2 ml) from frozen stocks was centrifuged for 10 min at $6000 \times g$ to remove autologous platelets. Platelets of a seronegative donor were adjusted to 4×10^8 /ml in phosphate-buffered saline (PBS). To cross-link virions to platelets, 250 µl of the platelet suspension was

incubated with platelet-free patient plasma and polybrene for 4 h at 37 °C in a 12-well culture plate (Nunc). The concentration of polybrene in the diluted plasma was 2 mg/ml unless otherwise stated. After the cross-linking step, samples were transferred to microtubes and platelets collected by centrifugation at $6000 \times g$ for 10 min. Platelets were washed once in 1 ml PBS and then resuspended in 900 μ l culture medium containing 20 mM HEPES buffer. Serial 1:3 dilution of this platelet suspension was performed and 150 μ l of the respective dilution was then co-cultured in quadruplicates with 1×10^6 stimulated CD8-depleted PBMCs in a 24-well culture plate (final volume was 1 ml). Viral replication was monitored by measuring p24 antigen production on days 7, 10, 14, 21, and 28 postinfection. Cultures were supplemented weekly with fresh medium and 2.5×10^5 stimulated healthy donor cells. TCID₅₀ was determined using the method of Reed and Muench (1938). Infectious titers reported refer to the infectious doses measured on day 14. Using an input of total 2 ml patient plasma in four replicate wells, the minimum infectious dose that can be detected in our assay is 2.29 TCID₅₀/ml.

To compare the newly developed assay with the commonly used method that employs direct co-culturing of patient plasma and target cells, titrations were set up using the protocol recommended by the Division of AIDS, National Institute of Allergy and Infectious Diseases for use in clinical trials (The Division of AIDS, National Institute of Allergy and Infectious Diseases and National Institute of Health, 1997).

Virus adsorption measured by p24 antigen deposition

Seronegative plasma (800 μ l) or PBS was spiked with HIV-1 isolates (approximately 10 ng p24/ml) and incubated with 1.25×10^8 platelets and the indicated concentrations of polybrene in PBS in a total volume of 1 ml. After incubation for 4 h at 37 °C, the samples were centrifuged at $6000 \times g$ and pellets washed once with PBS. Then, platelets were resuspended in 800 μ l seronegative plasma (diluted 1:2 in PBS), attached virions lysed by addition of 5% Empigen (Calbiochem; 200 μ l), and p24 content measured by ELISA. Mock-treated platelets were used to obtain background levels. All samples were measured on the same ELISA plate in duplicates and background subtracted. The 100% values (corresponding to the p24 content of the spiked virus) were determined by measuring p24 antigen concentration in a mix of spiking virus, plasma, and 200 μ l of 5% Empigen in a total volume of 1 ml.

Virus adsorption measured by real-time PCR

Viral RNA was quantified as follows: virus from plasma specimens was concentrated by high speed centrifugation at $50000 \times g$ and extracted using the RNAeasy mini kit (Qiagen Hilden, Germany). Platelet-bound viral preparations were extracted by a protocol utilizing phenol extraction and ethanol precipitation (RNAagents, Promega,

Mannheim, Germany) because polybrene in these samples precluded binding to silica matrices (data not shown). Genomic HIV-1 RNA was amplified in an i-cycler real-time thermocycler (Biorad, Lucerne, Switzerland) and quantified by comparison to an external standard curve utilizing synthetic HIV-1 RNA. A highly sensitive one-tube RT-PCR protocol in which cDNA synthesis and PCR reagents are separated by a wax layer and reunited by incubation at high temperatures was used as previously described (Ros-cic-Mrkic et al., 2003) utilizing the Qiagen one-step PCR kit. First-strand cDNA synthesis was accomplished with the primer skcc1b (Christopherson et al., 2000). PCR primers and the fluorescent probe for real-time detection of amplified DNA that mapped to a highly conserved region in the gag gene skcc1b (Christopherson et al., 2000) were described previously (Fischer et al., 2003).

Phylogenetic analyses of envelope fragments

RT-PCR, cloning, and sequencing of a HIV-1 *env* fragment encompassing C2 and V3 were performed as described previously (Fischer et al., 2004). Viral RNA was obtained directly from plasma and patient PBMC and also from viral isolates derived from plasma and PBMC cultures. Sixteen clones per sample were analyzed. Sequences were manually edited and aligned with Lasergene software version 5.06 (DNASTAR Inc., Madison, WI). Distances were calculated by MEGA version 2.1 (S. Kumar et al.; <http://www.megasoftware.net>) assuming a Tamura–Nei substitution model. Phylogenetic trees were constructed for each patient separately by maximum-likelihood method using T/T ratio 2.0, 10 replicate rounds of randomized sequence input, global rearrangement option, and HXB2 as reference sequence (PHYMLIP version 3.6; J. Felsenstein, University of Washington, Seattle; <http://evolution.genetics.washington.edu/phymlip.html>). Trees were displayed with TreeView (version 1.6; R. Page, University of Glasgow, UK; <http://taxonomy.zoology.gla.ac.uk/rod/treeview.html>). The clonal sequences were grouped into distinct phylogenetic clusters if they were separated by a branch having a length significantly different from zero. Branch lengths were defined to be significantly positive when zero was outside the confidence interval. Sequences differing by less pronounced distances were considered to belong to the same quasispecies.

Nucleotide sequence accession number

All C2-V3 *env* sequence described in Fig. 5 have been deposited in Gene Bank under accession numbers AY656249–AY656581.

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